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"Development of Regulatory Processes in the Symbiosis Between the Sea Anemone *Aiptasia pallida* and its Dinoflagellate Symbionts"

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Clayton B. Cook
Fredric Lipschultz

Bermuda Biological Station for Research
17 Biological Station Lane
Ferry Reach GE01
BERMUDA

Harbor Branch Oceanographic Inst.
5600 US 1 N.
Ft. Pierce, FL 34946
[CBC]

Office of Naval Research
800 N. Quincy St.
Arlington, VA 22217-5000

Distribution unlimited

Laboratory populations of the sea anemone *Aiptasia pallida* and other symbiotic marine invertebrates were used to investigate how symbiosis affected both dinoflagellates (zooxanthellae) and their hosts. Studies included the infection of algae-free hosts, responses to "host factors", metabolism of ^{15}N-ammonium and other aspects of how nitrogen was utilized by the symbiotic systems. Zooxanthellae of *A. pallida* showed distinct reposes to symbiosis: symbiotic cells were highly infective in host tissue and responded to host factors by releasing significant amounts of photosynthetic carbon. Cultured cells were only sparingly infective, and responded to host factors with reduced release of photosynthetic products. These effects were reversed following growth in host tissue. Preliminary characterization of host factors from a variety of marine hosts indicated small molecules with molecular weights of 3,000 or less. Both symbiotic algae and host tissue assimilated ^{15}N-ammonium, but there was little evidence for the recycling of this nitrogen between animal tissue and the symbionts. Algae-free animal tissue was capable of ammonium assimilation.

Symbiosis, zooxanthellae, dinoflagellates, sea anemones, corals

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SUMMARY OF RESEARCH
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The goal of this research was to investigate control mechanisms in symbioses between symbiotic dinoflagellates ("zooxanthellae") and marine hosts, particularly in the sea anemone *Aiptasia pallida*. We asked questions which focused on how physiological and molecular aspects of the host and the algae were affected by the symbiotic condition. These included the action of molecules ("host factors") in host tissue which affect the algae, the infection of anemones by algae, and aspects of nitrogen metabolism of the symbiosis including the metabolism of ^{15}N-ammonium.

(A) Host factor work: (CBC)

"Host factor" activity, originally discovered by Muscatine (1967) in coral and giant clam tissue, stimulates the release of short-term (< 1 h) photosynthetic products from symbiotic dinoflagellates. We concentrated on three aspects of this phenomenon: (1), the effects of symbiosis on the activity of host tissue; (2), the effects of symbiosis on the responses of the algae, and (3) characterization of the factor(s) from various hosts. Given the diversity of dinoflagellates which occur as symbionts with marine invertebrates, we restricted our work to the symbionts which occur in the sea anemone *Aiptasia pallida* (Bermuda strain). CBC has maintained clones of both the anemone and the cultured symbiont for over ten years. While tissues from various hosts were used in our studies, only freshly isolated symbionts ("FIZ") and the cultured algae ("CZ") were used in this work. FIZ from the anemones respond to preparations from a number of host species.

In these experiments, suspensions of algae (FIZ or CZ) were incubated with samples of host tissue (or filtered seawater for controls) with ^{14}C-bicarbonate in microfuge tubes for 30-40 min. Acidified samples of cells plus medium, and of medium only, were used to determine the release of fixed C to the medium. Typical seawater release by FIZ under these conditions would range from 2 - 5% of total fixed C (cf. data below).

The effects of symbiosis on the activity of host tissue. Trench (1971) reported that host factor activity in the sea anemone *Anthopleura elegantissima* required the presence of symbionts in host tissue. We found no such effect in *Aiptasia pallida*: aposymbiotic anemones, which have been maintained free of symbionts for over ten years, exhibited the same tissue-specific-activity as symbiotic anemones (Fig. 1; Cook and Orlandini, 1994). In a preliminary study, we obtained samples of the coral *Montastrea annularis* from the Florida Keys which had been bleaching (losing symbionts) for a period of two months. Normal tissue from this coral stimulated FIZ to release 15.30% ± 0.20% (n = 2), while bleached tissue effected a release of 14.6% ± 1.17% (n = 3) of fixed C. The loss of symbionts over this short period appeared to have little effect on host factor activity.

Thus, our work indicates that host factor activity in *A. pallida* and *M. annularis* is not a response to the possession of algal symbionts.

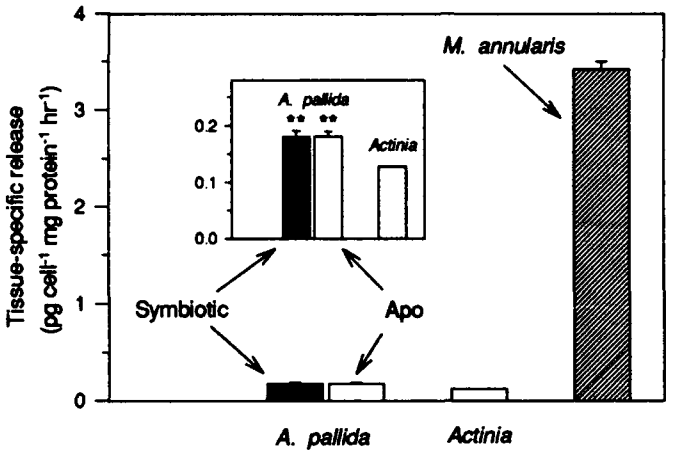

Figure 1. Tissue specific levels of host factor activity in symbiotic and aposymbiotic *Aiptasia pallida* and the reef coral *Montastrea annularis*. (Cook and Orlandini, in prep.)

The effects of symbiosis on the responses of the algae. CZ and FIZ have strikingly different responses to host factor samples. While FIZ have enhanced release of short-term photosynthetic products, CZ display significantly reduced release (Fig. 2A, 2C). FIZ lose the sensitivity to HF soon after isolation from host tissue. Fig 2B summarizes an experiment in which FIZ are isolated and inoculated into the liquid culture medium used to grow CZ. Within 24 h of isolation, the host factor response is lost. In the converse experiment, CZ were injected into aposymbiotic tissue, and the algae isolated and tested for sensitivity at intervals thereafter. The algae respond to host factor within two weeks of infection, but do not exhibit complete response until 5-8 weeks of residence in host tissue (Fig 2C; Cook and Olson, in prep.). In Figure 2C the data are plotted as the ratio of release rates in host factor and filtered seawater (FSW). Note that in every test of the CZ algae, this ratio is less than 0.5 -- that is, there is apparently less release by these algae in host factor than in seawater alone.

These results show that the response of *A. pallida* symbionts to host factor is affected by the symbiosis: algae which grow in host tissue exhibit the response, while those which are grown in culture do not. Thus, host factor sensitivity is a genetic response to the symbiosis.

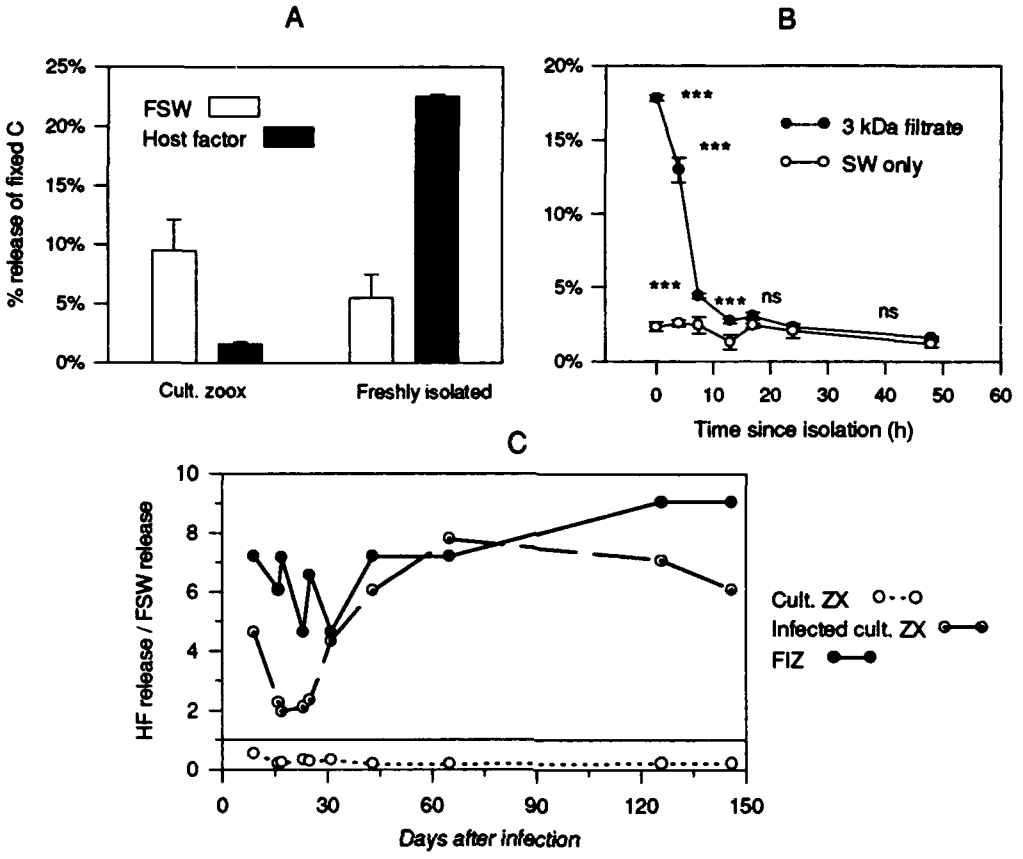

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Figure 2. Differences in sensitivity of cultured zooxanthellae (CZ) and freshly isolated zooxanthellae (FIZ) to host factor (3 kDa filtrate from *Montastrea annularis*). A. Typical experiment showing the differences in responses. B. Changes in FIZ following inoculation into liquid culture. Statistical comparisons of FSW versus host factor from Tukey HSD tests: ***, $p < 0.001$; ns, not significant. C. Responses of CZ following infection into aposymbiotic *A. pallida* tissue. All tests were conducted using the same 3 kDa filtrate preparation. Note that ordinate plots the ratio of HF to FSW release, hence values > 1 indicate stimulation. (Cook and Olson, in prep.)

Preliminary characterization of host factor material. We examined host factor material from a variety of zooxanthellate hosts from Bermuda, and concentrated on *Aiptasia pallida* and the reef coral *Montastrea annularis*. Fig. 1 shows that the tissue-specific activity from this coral can be more than 20 times higher than that of the anemone; hence extracts of this coral were used in many of our experiments (cf., Figs. 2b,c).

Our initial characterization involved ultrafiltration. This size fractionation showed that all host factor activity from *A. pallida* (both symbiotic and aposymbiotic), three corals (*M. annularis, Oculina diffusa* and *Agaricia fragilis*), a jellyfish (*Cassiopea xamachana*) and a hydroid (*Myrionema amboinense*) was less than 3 kDa in molecular weight. This contrasts with the one previously published report of host factor activity

in an Australian coral with a molecular weight > 10,000 (Sutton and Hoegh-Guldberg, 1990).

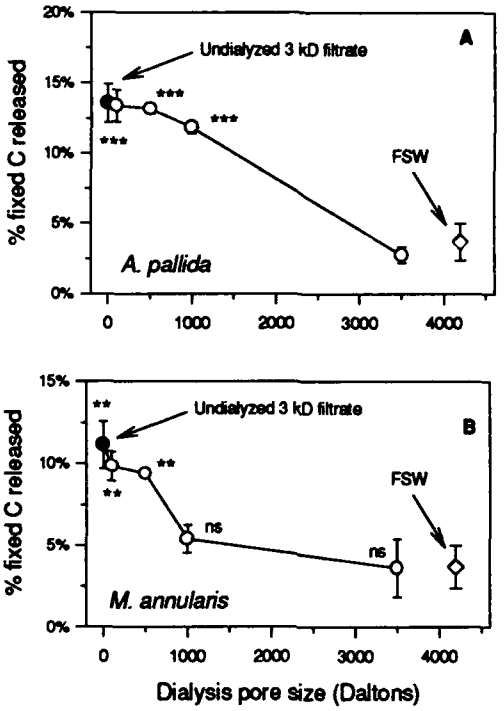

Figure 3. Molecular fractionation by dialysis against filtered seawater of 3 kDa filtrates of (A) *Aiptasia pallida* and (B) *Montastrea annularis*. (Cook and Orlandini, in prepr.)

Dialysis of 3 kDa filtrates from *A. pallida* and *M. annularis* indicated that the material from the anemone was <1 kDa in size, while that from the coral was between 500 and 1000 Da (Fig 3; Cook and Orlandini, 1994). Curiously, material from the anemone -- apparently of somewhat larger size -- was not affected by boiling, while that from the coral lost 75% of its activity by this treatment (Fig. 4). Boiled coral samples still had more activity than did seawater alone. These results indicate that different molecules -- and perhaps different molecular mechanisms -- are involved in the two systems.

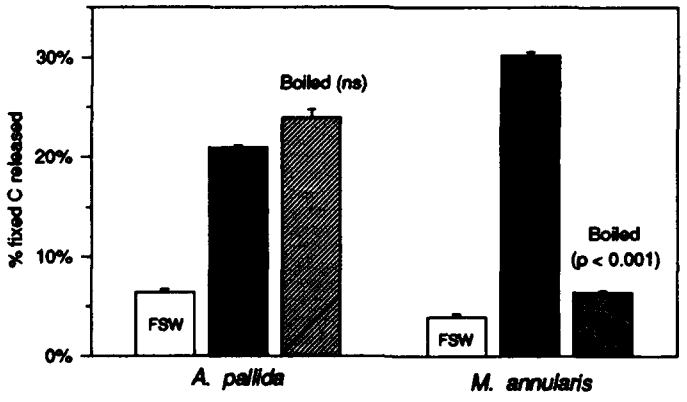

Figure 4. The effect of boiling for 30 minutes on crude extracts of *Aiptasia pallida* and *Montastrea annularis*

(B) Infection of anemones by cultured and freshly isolated zooxanthellae (CBC).

Another major difference that we found between freshly isolated zooxanthellae (FIZ) and cultured zooxanthellae (CZ) was in growth following infection into host tissue. We assayed growth by direct observations of algae in living tentacles. When FIZ were injected into the guts of algae-free anemones, the gut cells became intensely brown within 24 hours indicating rapid endocytosis of algae. These algae became a source of recruitment into tentacle cells. In contrast, cultured *A. pallida* symbionts were taken up only very sparingly by gut cells, so that initial accumulation in tentacles occurred largely through direct endocytosis by tentacle cells. Thus the effective growth rate of injected FIZ in tentacles exceeded that of all other experimental groups (Table 1). However, FIZ still had greater growth rates than CZ when the algae were merely added to experimental dishes, and not injected into the gut. The growth of CZ in tentacles was not significantly different from the growth of CZ in test tubes.

These observations suggest (a) that cell surface differences between CZ and FIZ may be responsible for differences in endocytosis, and (b) that over the period of the observations, adjustments of CZ must still be made -- perhaps including the sensitivity to host factor discussed above-- before growth rates comparable to FIZ are achieved.

Table 1. Growth rates of *Aiptasia pallida* symbionts in tentacles of *A. pallida* during infection. Each value (± s.d.) is the mean of 3-5 replicates.

Source of algae	Doubling time (days)	Specific growth rate (doublings day^{-1}
FIZ (injected)	1.1 ± 0.1	0.92 ± 0.12
FIZ (free-swimming)	2.1 ± 0.8	0.76 ± 0.18
CZ (injected)	3.5 ± 1.2	0.31 ± 0.08
CZ (growth in tubes)	4.3 ± 0.2	0.24 ± 0.03

(C) Assimilation of ^{15}N ammonium (FL)

The objective of this ^{15}N work was to trace the movement of nitrogen between zooxanthellae and the animal host. The time course of ^{15}N distribution in various metabolic compartments should indicate if host tissue is involved in ammonium assimilation, and the degree of recycling of nitrogen between host and zooxanthellae. Our strain of *Aiptasia pallida* (symbiotic and aposymbiotic) was used for most experiments. A much larger anemone, *Bartholomea annulata*, was also used to permit repeated sampling of the same individual over time. The basic experimental design was a short, pulse exposure to $^{15}NH_4$ followed by a longer chase period with unlabeled, low nutrient seawater to allow the label to redistribute among the various operationally defined metabolic compartments. At various times, zooxanthellae and host tissues were separated and then fractionated into macromolecular and low molecular weight components. The concentration and isotopic composition of NH_4 in the external media was measured to quantify uptake and to determine if concurrent excretion was occurring.

As expected, there was net uptake of NH_4 by symbiotic anemones but not by animals without zooxanthellae. Isotope dilution during the pulse period and excretion of $^{15}NH_4$ during the 10 day chase period demonstrated concurrent excretion of ammonium. Ammonium inside the animal came to equilibrium with the external label after 4 hours and then was rapidly diluted at the beginning of the chase period. Dilution then slowed after 24 hrs until a low degree of labeling was reached that remained constant over the ensuing 10 days.

The pattern of labeling in the low molecular weight (LMW) components of both zooxanthellae and animal in *B. annulata* closely mirrored the pattern observed for the ammonium pool. These results suggest that rapid cycling of nitrogen occurs between the two LMW compartments. However, the rapid dilution during the chase requires a source of unlabeled nitrogen such as "old" protein that is being degraded or is turning over. Loss of unlabeled macromolecules would result in an increased ^{15}N abundance in the remaining macromolecules.

It is interesting that the macromolecules in the zooxanthellae and host tissue do not change after the first few hours of the chase period, and retain a far higher isotopic composition than their respective LMW pools. The macromolecular compounds do not appear to supply the required unlabeled nitrogen to dilute the LMW, and their higher isotopic composition does not produce increased of 15N in the LMW! Rather, the constant composition of these compartments implies a lack of interaction between the zooxanthellae and animal macromolecules, and conservation rather than recycling. At this point, the nitrogen source causing the dilution of the LMW and NH_4 pools is unknown.

The effect of feeding versus starvation was tested using *A. pallida* that were exposed to $^{15}NH_4$ for 7 days. During the chase period, individuals were either fed brine shrimp each day for 10 days or starved. In contrast to the short (4-8 hr) pulse experiments, the isotopic composition of the macromolecules in the zooxanthellae declined 50% over the 10 days, but the feeding regime had no effect. The isotopic composition of the animal fraction remained constant as in the other experiments with no evidence of interaction with the more highly labeled algal macromolecules. The dilution of the zooxanthellae in this experiment is likely due to enhanced growth stimulated by the 7 day exposure to ammonium. The lower specific activity animal nitrogen must have fueled the dilution during the chase suggesting a physiological shift in regulation of nitrogen recycling.

The rapid appearance of ^{15}N in macromolecules during the pulse argues for close coupling to the respective LMW pools of both animal and alga. In contrast, the constant composition of the macromolecules during the chase while the LMW composition declines suggests little interaction. The pulse exposure to 10 μM NH_4 might temporarily foster a biomass increase that ceases during the chase; recycling followed by conservation of nitrogen?

(D) Other aspects of nitrogen utilization in zooxanthellae symbioses: (CBC)

We used a variety of techniques to study how nitrogen was utilized in other symbioses with zooxanthellae. The enhancement of dark carbon fixation by ammonium is commonly used by phytoplankton biologists to assess the nitrogen status of natural populations. We used this approach to demonstrate that feeding by corals makes

zooxanthellae more nitrogen-sufficient, and that these algae are more likely to be nitrogen-deficient when they exist at dense populations in host tissue (Cook et al., 1994). By analyzing intracellular pools of glutamate and glutamine and comparing these with cellular growth data, we demonstrated that zooxanthellae symbiotic with the hydroid *Myrionema amboinense* became severely N-deprived when the host has not fed, but this effect could be ameliorated by the addition of ammonium to seawater (McAuley and Cook, 1994). In a similar fashion, the long-term addition of ammonium to the Hawaiian coral *Pocillopora damicornis* resulted in elevated N-content of zooxanthellae compared to untreated control corals, although the N-content of host tissue was not affected (Muller-Parker et al., 1994). These studies complement our work with ^{15}N, and indicate that "recycling" of nitrogen within these symbioses may not be as extensive as previously thought.

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LIST OF PATENTS PENDING OR FILED

(None)